

A 52 Kilodalton Protein Vaccine Candidate for *Francisella tularensis*

C.A. Sikora, B.J. Berger and J.W. Cherwonogrodzky
Defence R&D Canada – Suffield

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This study was conducted in conformity with the Guide to the care and use of experimental animals, published by the Canadian Council on Animal Care.

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Abstract

For identifying *Francisella tularensis* vaccine candidates, mice were first vaccinated with *Brucella abortus* O-polysaccharide (OPS) vaccine. These animals were then given 10 LD₅₀s of *F. tularensis* live vaccine strain (LVS). Sixty percent (60%) of the vaccinated mice survived the multiple lethal dose while all the unvaccinated control mice perished. Sera were collected from these surviving mice and used to probe supernatant and cell lysates of live *F. tularensis* LVS cultures. Several *Francisella tularensis* components were identified by this noted antiserum. Mouse serum from mice vaccinated with killed *F. tularensis* did not identify these components. Of these identified proteins, enzyme digestions and chemical oxidation suggest post-translational modifications for some of the proteins (e.g. a 52 kilodalton (kDa) glycoprotein, a 45 kDa lipoprotein and a 19 kDa nucleoprotein). In low concentrations, the 52 kDa component caused nitrous oxide induction in tissue cultures and in high concentrations it caused cell death. Vaccination with this protein gave mice partial protection (20% survival) from 250 LD₅₀ of tularemia given intranasally while the addition of other components may have acted synergistically to give enhanced protection (i.e. 100% survival).

Résumé

Pour identifier les candidats au vaccin *Francisella tularensis*, des souris ont d'abord été vaccinées avec le vaccin O-polysaccharide *Brucella abortus* (OPS). Ces animaux ont reçu 10 DL⁵⁰ de la souche du vaccin vivant *F. tularensis* (LVS). Soixante pourcent (60%) des souris vaccinées ont survécu la dose létale multiple alors que toutes les souris non vaccinées du groupe témoin ont péri. Des sérums ont été recueillis à partir des souris qui ont survécu et ont été utilisés pour sonder le surnageant et le lysat des cellules de cultures vivantes du vaccin vivant *F. tularensis*. Plusieurs composants de *Francisella tularensis* ont été identifiés par cet antisérum. Le sérum de souris provenant de souris vaccinées avec *F. tularensis* non-vivant n'a pas identifié ces composants. A partir de ces protéines identifiées, les digestions d'enzymes et l'oxydation chimique suggèrent des modifications post traductionnelles pour certaines des protéines (par ex. : une glycoprotéine de 52 kilodaltons (kDa), une lipoprotéine de 45 kDa et une nucléoprotéine de 19 kDa). Dans les faibles concentrations, le composant de 52 kDa a causé l'induction d'oxyde de diazote dans les cultures de tissus et la mort de la cellule, à des hautes concentrations. La vaccination avec cette protéine a procuré une protection partielle pour les souris (20 % ont survécu) avec 250 DL⁵⁰ de tularémie donnée par voie intranasale alors que les autres composants ajoutés ont pu agir synergiquement pour résulter en une meilleure protection (ex : 100% ont survécu).

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Executive summary

The presented study provided a strategy for the development of safe and effective sub-cellular vaccines against biological threat agents, such as tularemia. The method used a vaccine against one threat agent (e.g. *Brucella abortus*) to protect mice from a different but cross-reactive threat agent (e.g. *Francisella tularensis* live vaccine strain). These vaccinated mice were then given multiple lethal doses (e.g. 10-250 LD₅₀) of the latter and were allowed to experience, survive and display an immune response to an otherwise lethal infection. Their antiserum was used to identify the bacterial virulence factors expressed during the active disease process.

A 52 kilodalton molecular weight (kDa) protein of *F. tularensis* was expressed in minor amounts as evidenced by Coomassie blue staining of polyacrylamide gels. In contrast, mice that had been vaccinated and survived multiple lethal doses of tularemia had antibodies in their sera that recognized this component as a prominent antigen, as shown in immunoblots. Periodate treatment followed by silver staining suggests that this protein was sometimes glycosylated. *In vitro* studies showed that in low amounts this protein caused nitrous oxide production in some mammalian cell lines and in high amounts it caused cell death. Vaccination of balb/c mice with this 52 kDa protein provided low protection (20% survival) against 250 LD₅₀ of LVS given intranasally. Enhanced protection (100% survival) was observed for co-vaccination with other *F. tularensis* cell components greater than 30 kDa and the *Brucella* O-polysaccharide.

That these mice had sera with antibodies that recognized previously overlooked components showed a novel method for finding vaccine candidates. The presence and expression of these components may also assist in the prediction of protective efficacy of different vaccine lots. That some of these components were specific to *Francisella tularensis* provided a means of differentiating the infecting agent, even in the presence of cross-reactions that currently confuse identification.

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Sommaire

Cette étude fournit une stratégie pour la mise au point de vaccins sub-cellulaires sécuritaires et efficaces contre les agents biologiques dangereux tels que la tularémie. La méthode utilise un vaccin contre un agent dangereux (par ex : *Brucella abortus*) pour protéger les souris d'un agent dangereux différent mais d'activité hétérospécifique (par ex : la souche du vaccin vivant *Francisella tularensis*). Ces souris vaccinées ont reçu les doses létales multiples (de 10 à 250 DL⁵⁰) de ce dernier et ont pu faire face, survivre et afficher une réponse immunitaire à une infection d'autant plus létale. Leur antisérum a été utilisé pour identifier les facteurs de virulence bactérienne exprimés durant le processus actif de la maladie.

Une protéine de *F. tularensis* d'un poids moléculaire de 52 kilodaltons a été exprimée en quantités minimes comme mis en évidence par la coloration en bleu de Coomassie des gels polyacrylamides. Par contre, les souris qui ont été vaccinées et survécu aux doses létales multiples de tularemia possédaient des anticorps dans leur sérum qui ont reconnu ce composant comme un antigène proéminent, tel que démontré dans les immunoblots. Les traitements périodiques suivis des colorations argentées suggèrent que cette protéine a été quelquefois glycosylée. Les études *in vitro* indiquent que cette protéine en faibles quantités cause la production d'oxyde de diazote chez certaines lignées cellulaires mammaliennes et qu'en grandes quantités, elle cause la mort de la cellule. La vaccination de souris balb/c avec cette protéine de 52 kDa a fourni une faible protection (20 % ont survécu) contre 250 DL⁵⁰ de souche de vaccin vivant (LVS) donné par voie intranasale. On a observé une protection améliorée (100% ont survécu) pour la co-vaccination avec d'autres composants de cellules *F. tularensis* supérieurs à 30 kDa et le O-polysaccharide *Brucella*.

Le fait que ces souris aient un sérum qui possède des anticorps capables de reconnaître les composants qui avaient été antérieurement négligés indiquent qu'il s'agit d'une nouvelle méthode capable de trouver les candidats au vaccin. La présence et l'expression de ces composants peuvent aussi faciliter la prédiction de l'efficacité de la protection des lots de vaccins différents. Le fait que certains de ces composants soient spécifiques à la *Francisella tularensis* est un moyen de différencier l'agent infectant, même en présence des réactions hétérospécifiques qui obscurcissent actuellement l'identification.

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Introduction

Tularemia is a pneumonic and systemic illness caused by the bacterium *Francisella tularensis*. It is primarily a disease of wildlife that spreads to humans incidentally. Transmission may be by insect or tick bites, handling infected carcasses or drinking contaminated water. The disease usually progresses from an ulcer at the site of infection to the rest of the body, notably the lungs, to cause a severe illness with a high mortality rate (30-60%) unless antibiotic therapy is given [1]. The bacterium is readily grown on simple medium, is highly virulent when delivered as an aerosol or in contaminated water and is a potential biological warfare or terrorist threat agent [2].

For medical countermeasures against tularemia, antibiotics can clear the infection, but the success of these antibiotics depends on where the infection has located in the body and how early the patient is treated. A *F. tularensis* live vaccine strain (LVS) is available, but it has only an Investigational New Drug (IND) status. Its efficacy against exposures by different routes of infection is questionable [3] and under some conditions it appears to revert to its virulent parental form [4]. There is, therefore, a need for a more effective safe vaccine against tularemia.

The identification of vaccine candidates against tularemia is not as simple as might be assumed. There are several thousands of bacterial proteins and the ones investigated to date have not proven useful [5]. One could use antibodies from the sera of either volunteers vaccinated with LVS or patients infected with field strains of *F. tularensis*, but there are inherent limitations. Vaccine strains may lack the virulence factors being sought and so vaccinated volunteers may lack antibodies to these. For infected patients, the reason that they are ill may be due to an inappropriate or ineffective immune response. Indeed, Edward Francis (after whom *Francisella* is named) had three infections of tularemia and eventually died from this disease [6]. The multiple infections did not provide him with an appropriate immunity against tularemia. What is required, therefore, is a means of expressing and identifying key components or virulence factors of *F. tularensis* that can serve as vaccine candidates to induce a protective response in the vaccinee.

For the first part (expressing virulence factors of the bacterium), it was previously found that the virulence of LVS can be enhanced 1000-fold by subculturing it in a synthetic salts medium [4]. However, it was subsequently found that this enhanced virulence was temporary, increasing with LVS being subcultured 3-5 times in the synthetic salts medium, but greatly diminishing with additional subcultures (unpublished results). It was also found that when the third broth subculture supernatant was filter-sterilized (0.2 µm filter), and 50 µl given intranasally to mice, the mice died a day later. These latter results (unpublished) suggested that a toxic agent was being produced by the bacterium, that it was being released into the supernatant of the culture and that it had a delayed action on the test animals.

For the second part (identifying virulence factors), other studies have suggested that toxins are not present for *F. tularensis* [7, 8]. However, in our study, when antiserum was used from *B. abortus* OPS vaccinated mice that experienced and survived the disease process of tularemia, an immunodominant 52 kDa protein of *F. tularensis* was identified. Results suggested that this 52 kDa protein may be a toxin that affects mammalian cells during the disease process of tularemia. The following study also investigates the possibility that the addition of other components to this 52 kDa protein may act synergistically to enhance the protection of mice from tularemia.

Materials and Methods

Bacterial Cultures

Francisella tularensis live vaccine strain (LVS) was acquired as a freeze-dried vaccine in a vial (Lot #11, Code Number: NDBR 101, 2.4×10^9 cfu/ml when rehydrated) from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, Maryland, USA. It was grown either on agar or in broth synthetic medium at 37°C, 90% humidity and 5% CO₂. For intranasal inoculation, a 10 LD₅₀ dose was 1 X 10⁵ bacteria diluted in 10 µl (prewarmed at 37°C) phosphate buffered saline (PBS). For intraperitoneal injections, a 10 LD₅₀ dose was 1 X 10⁶ bacteria in 100 µl PBS.

Brucella abortus strain 30 was acquired from the Animal Diseases Research Institute – Nepean, Nepean, Ontario. This was maintained on *Brucella* agar with 1.5 ppm crystal violet and incubated as for LVS. This culture was used to infect mice (within DRDC-Suffield Biocontainment Level 3 facilities) for sera against live *Brucella abortus*.

Two-percent phenol-killed *B. abortus* 1119-3 was acquired from Dr. Janet Payeur (USDA, Ames, Iowa). These cells were used as the source for O-polysaccharide vaccine, which was purified by a method previously described [9].

Bacterial Growth Media

For *F. tularensis* LVS, Chamberlain's synthetic medium base [10] consisted of 10g/L NaCl, 4g/L glucose, 1g/L (each) KH₂PO₄ and K₂HPO₄, 2g/L (each) L-proline and DL-threonine, 0.4g/L (each) of L-arginine, L-asparagine, DL-isoleucine, L-leucine, L-lysine HCl, DL-methionine, DL-serine, L-tyrosine and DL-valine, 0.2g/L (each) L-cystine HCl and L-histidine, 0.135g/L MgSO₄.7H₂O, 0.40 mg/L spermidine phosphate, 4 mg/L thiamine HCl, 2 mg/L DL-calcium pantothenate and FeSO₄.7H₂O (in triple distilled water, filter sterilized, pH 6.5). For broth cultures, a vial of stock culture was taken from the -70°C freezer, partially thawed and one loopful was used to inoculate 10 ml peptone-cysteine broth tubes. These tubes were incubated for a few days until growth was obvious and were then added to 500 ml synthetic medium base in 1 litre sterile flasks and incubated to early logarithmic or late stationary phases (shaken at 150 rpm, 37°C). For agar plates, double strength synthetic medium was made and placed in a 50°C water bath, a 4% agar (Difco Laboratories, Detroit, Michigan) in distilled water suspension was autoclaved (30 min, 121°C, 15 psi), cooled to 50°C, then these were combined and used to make pour plates. Plates were inoculated with the above noted peptone-cysteine culture. Subculturing involved transferring 1 ml of broth culture or a loopful of agar culture to the next sterile medium.

Brucella abortus 30 was maintained on *Brucella* agar (Difco Laboratories, Detroit, Michigan) with 1.5 ppm crystal violet and subcultured in *Brucella* broths (without crystal violet) at 37°C, 5% CO₂ and 150 rpm.

Sonication

For sonicating suspensions of cells, a Soniprep 15 and a Process Timer (both manufactured by MSE Ltd., Crawley, Sussex, UK) were used within a certified biosafety cabinet. Samples were in plastic tubes which were held with a clamp and partially immersed in ice-water in a small beaker. The amplitude of the 10 mm probe was adjusted manually to 10 µm, the sequence was 5 cycles of 15 second pulses followed by 1 min chilling. For the sonication of a live culture of *F. tularensis* LVS, two 500 ml cultures were centrifuged (10,000 X G, 4°C, 30 min), the white pellet was resuspended in 75 ml of sterile PBS then stored frozen at -70°C. Prior to use, this sample was thawed at room temperature (22°C). Sonication on these freeze-thawed stressed cells was as before except that 10 ml aliquots and 12 cycles were used for a total homogenization time of 3 min. The disrupted cell suspension was centrifuged (10,000 X G, 30 min) then the supernatant of the cell lysate was saved.

Use of Animals (Mice)

Mice (BALB/c or CD1, female, 19-21 g, 35 days old) were acquired from Charles River (St. Constant, Quebec). Mice were cared for in accordance with the guidelines set by the Canadian Council for Animal Care and all procedures were reviewed and approved by the DRDC Suffield Animal Care Committee. To raise anti-tularemia mouse sera, twenty mice were first vaccinated with the *B. abortus* O-polysaccharide (OPS). Each mouse was given 1 µg OPS in 0.1 ml sterile saline by the intraperitoneal route (i.p.). Two weeks later, these mice were taken to a BioSafety Cabinet and infected i.p. with 10 LD₅₀ of *F. tularensis* LVS diluted in sterile prewarmed PBS. Infected mice were then placed into isolator cages (with adequate food and water, with HEPA filter tops) which in turn were placed into a HEPA-filtered Animal Isolator (Thoren Caging, Toronto). The mice were monitored for 2 weeks. Sixty percent of the OPS vaccinated mice survived 10 LD₅₀ of *F. tularensis* LVS and these were sacrificed for their anti-tularemia serum. For infection by the intranasal route, mice were anaesthetized with 100 µl of 1:10 diluted Somnotol (MTC Pharmaceutical, Cambridge, Ontario) given intraperitoneally. Once the mice were unconscious, 10 µl of the bacterial suspension was delivered to a nostril of each mouse by a micropipette tip.

Sera

Blood from each mouse (100-300 µl) was collected by inserting a 26 gauge needle into a prewarmed tail vein and then transferring the blood to a 1.5 ml small micro-centrifuge tube. Larger volumes (1 ml) were collected by first anaesthetizing mice with a double dose of Somnotol, then performing a heart-puncture with a 1 ml syringe and 26 gauge needle. The blood was centrifuged (5,000 X G, room temperature, 10 min) and the serum transferred to a labeled centrifuge tube. Glycerol was added to the serum for a final concentration of 10%. Most of the methods used fresh serum. It was later found that serum that was stored frozen at -70°C for extended times did not yield the same specific results as when the serum was used fresh.

Polyacrylamide Gel Electrophoresis (PAGE)

For the resolution of low molecular weight components, 12% polyacrylamide gels (reagents from Sigma Chemical Co., St. Louis, Missouri) were prepared using the method of Laemmli [11]. For separating components of higher molecular weight, a 7.5% polyacrylamide gel was used. Electrophoresis was performed in a Bio-Rad minigel system (Bio-Rad Laboratories Ltd., Mississauga, Ontario) following the protocols outlined by the manufacturer. For electrophoresis, the electrolyte running buffer was made by combining 3 g/L Tris base, 14.4 g/L glycine and 1g/L SDS (SDS was not used for native gels) in 1 litre of triple distilled water.

A double-strength buffer for samples consisted of 100 mM Tris-HCl (pH 6.8) and 200 mM dithiothreitol (DTT) with 20% glycerol, 2% SDS (absent for native gels) and 0.1% bromophenol blue. Samples were diluted 1:1 with sample buffer, boiled for 10 min, vortexed for 5 seconds, cooled, then centrifuged for 10 seconds at maximum speed (12,000 rpm, 13,000 X g) in a micro-centrifuge to remove denatured debris. A total of 10 µl was transferred to each well in the gel. Samples were run at a constant voltage of 100 V and duplicate gels were run concurrently for a protein stain and an immunoblot.

For proteins, polyacrylamide gels were stained with a solution of 0.025% Coomassie Brilliant Blue R250, 40% methanol, 7% acetic acid and 53% water. Destaining was done with several changes of 40% methanol, 7% acetic acid and 53% water. Once destained, the gels were stored in 5% glycerol in water. Alternatively, the Bio-Rad (Mississauga, Ontario) silver stain was used with dichromate as the oxidizer. Molecular weight standards (Bio-Rad Laboratories, Mississauga, Ontario) were myosin (200 kDa), beta-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anydrase (31 kDa), soybean trypsin inhibitor (22 kDa), lysozyme (14 kDa) and aprotinin (6.5 kDa).

To stain lipopolysaccharide (LPS) within these gels, the Bio-Rad (Mississauga, Ontario) silver stain was used with periodic acid to oxidize the carbohydrates.

Western Immunoblots

Following electrophoresis, the gel was electro-blotted onto a Hybond nitrocellulose membrane (Amersham Pharmacia Life Sciences, Baie Dorfe, Quebec) in a mini Protean-2-electrotransfer apparatus (Bio-Rad, Mississauga, Ontario). The transfer buffer was 3 g of Tris-base (Sigma Chemicals, St. Louis, USA) in 14.4 g/L glycine and 200 ml/L of methanol in water. To block binding sites on the nitrocellulose membrane, the membrane was placed in a 5% solution of ECL blocking agent (Amersham Pharmacia Life Sciences) and phosphate buffered saline with 0.1% Tween-20 (PBS-Tween) for an hour at room temperature (22°C) with gentle mixing. The membrane was then washed for 10 minutes in PBS-Tween20, followed by three subsequent washes of 5 min each. Serum (from control unvaccinated mice, mice infected with *B. abortus* 30, mice vaccinated then infected with 10 LD50 of *F. tularensis* LVS, or rabbit anti-*F. tularensis* antiserum) was the primary antibody diluted 1:1000 in PBS-Tween. The nitrocellulose membrane was incubated in 50 ml of this antibody solution for 1 hour in PBS-Tween as before. The secondary antibody was a Caltag anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (Cedarlane Laboratories Ltd., Hornby, Ontario), diluted 1:3,000 in 50 ml of PBS-Tween and incubated at room temperature for an hour with gentle swirling. The membrane was then washed in PBS-Tween as noted above.

Following the last wash, the membrane was placed onto a clean sheet of acetate, ECL solutions were applied, a second acetate was layered onto this and the preparation was processed as per the manufacturer's instructions (Amersham Pharmacia Life Sciences). The immunoblot was used to expose ECL Hyperfilm (ECL Chemicals, Amersham Pharmacia Life Sciences, Baie Dorfe, Quebec) for 40 minutes. The latter film was developed in Kodak GBX developer, fixed for 3 minutes, washed in distilled water for 5 minutes, dried overnight and then photographed.

Protein Determination

Protein content of test samples was assayed by using the Bradford protein assay (Bio-Rad Laboratories) with detection at 595nm in a Thermomax microtitre plate reader (Molecular Devices, Sunnyvale, CA). The results were analyzed with the software program, Softmax™ (also by Molecular Devices).

Sample Preparations

Francisella tularensis LVS cultures grown in broths for 3 days. Each culture was centrifuged, the supernatant removed and filter-sterilized through a 0.22 µm Nalgene filter sterilizing flask (1 litre). Part of the supernatant volume was stored in the refrigerator, part was freeze-dried. The bacterial pellet was resuspended in PBS, frozen at minus 70°C then thawed when required and sonicated as noted in the previous section.

Cultures were grown on agar plates for a week. The cells were then scraped off the agar surface, the agar from a plate was cut into pieces, transferred to a plastic bag, 50 ml of sterile PBS was added, the bag sealed and then contents crushed on a Stomacher (Seward, UK) for 3 minutes. The bag was then opened, the slurry transferred to a centrifuge bottle, this was centrifuged (10,000 X G, 4°C, 30 minutes), and the supernatant was removed then filter-sterilized as before. Part of this latter supernatant was stored in the refrigerator, part was freeze-dried.

Bacterial cell sonicates, with components released into PBS, were separated from cell debris by centrifugation (10,000 X G, 4°C, 30 minutes). The liquid (referred to as cell lysate) was placed into a Spectrapor 10,000 m.w. cutoff dialysis bag and dialyzed overnight at 4°C against 1mM HEPES and 1 mM DTT.

As required, the components of these various supernatant preparations were concentrated using an Amicon ultrafiltration membrane (Millipore, Nepean, Ontario) with a 30,000 m.w. cutoff.

Mammalian Cell Culture Viability and Nitric Oxide Determination

HeLa, Vero and the J774.1 cell lines were obtained from American Type Culture Collection (ATTC, Manassas, Virginia, USA). HeLa or Vero cells were grown in a T25 Corning Tissue Culture Flask (Fisher Scientific, Ottawa, Ontario) with 5 ml of Dulbecco's Modified Eagle Medium supplemented with fetal calf serum, MEM amino acids, L-glutamine, sodium pyruvate, MEM vitamins and anti-bacterial/antifungal antibiotics (InVitrogen, Burlington, Ontario). Incubation was at 37°C with 5% CO₂ and growth to a confluent layer was confirmed visually with an inverted microscope. J774.1 was grown in sterile 96-well tissue plates using modified minimal essential medium (mMEM, Flow Laboratories, Mississauga, Ont.) without phenol red, supplemented with streptomycin (10µg/mL), penicillin (10 U/ml), fungizone (0.25 ug/ml) and 5% horse serum. Initial concentration of macrophages was about 5000 cells/well. As a control and blank, the bottom row did not have cells. Volumes of 50 µl fresh medium and 50 µl serially diluted test sample were added to the wells. After 24 hrs, the media were transferred to a fresh microtitre plate and an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphylethylenediamine dihydrochloride, 2.5% H₂PO₄) was added for nitrate determination [12]. This mixture was incubated at room temperature for 10 minutes and then read at 540 nm in a microtitre plate reader (Molecular Devices). Sodium nitrate was used as a standard for nitrate quantitation.

Concurrently, cell viability was assessed using AlamarBlue (AccuMed International Inc., Westlake, OH). One hundred microlitres of a 10% (v/v) concentration was added to each well. The plate was incubated for 5 hours at 37°C, 90% humidity, 5% CO₂. The absorbance (570 nm) was read on a Thermomax titre plate reader (Molecular Devices); the bottom row without cells served as the control and blank.

Column Chromatography Fractionation/Purification

The 52 kDa protein in cell lysates and freeze-dried supernatants from *F. tularensis* LVS cultures was purified by column chromatography using a Pharmacia FPLC (Amersham Pharmacia Life Sciences). The column used was a Pharmacia Mono-Q 10/10 column. Buffer A comprised of 10 mM HEPES, Buffer B contained 10 mM HEPES and 3 M KCl. To elute bound components from the column, a gradient was programmed to raise the percentage of B-buffer to 50% over a time of 20 minutes, following the loading step. Elution was monitored at 280nm using a spectrophotometric detector. To increase binding, samples were dialyzed overnight in a Spectrapor 8 kDa molecular weight cut-off membrane against triple-distilled water.

Results and Discussion

Correlation Between Bacterial Subculture, Virulence and Release of Proteins

In a previous publication, it was reported that the subculture of *F. tularensis* LVS in a weakly acidic synthetic salts medium enhanced its virulence 1000-fold [4]. At the time, it was hypothesized that the enhanced virulence of the bacterium was associated with the observed enhanced capsule formation on the bacterium. However, after the publication, it was found that although the virulence of *F. tularensis* LVS was lost after the fifth subculture, its extensive capsule formation remained unchanged (unpublished results). The virulence of the bacterium, therefore, did not correlate with capsule formation.

When mice were infected, it was also found that the virulence of the bacterium increased from the first to the third subculture (when grown on either broth or agar synthetic media) but diminished after the fifth subculture (unpublished results). It was also found that, from the third subculture, 50 µl of filter-sterilized supernatant (free of the bacterial cell) given intranasally to mice caused death after 1-2 days (unpublished results). The culture medium were therefore inspected for extra-cellular material released from *F. tularensis* LVS. As the medium was synthetic salts medium lacking proteins, any proteins that were found would be from the bacterium. The broth supernatants or agar extracts were concentrated about a hundred-fold by lyophilization, reconstituted in distilled water and applied to a 12% SDS-PAGE. In our hands, the Coomassie stain for proteins was not sensitive enough to detect the low amount of proteins present in these concentrated samples (data not shown). A more sensitive silver stain for proteins was used and indeed extracellular proteins were detected with this method. A correlation was evident between the virulence of the subculture and the appearance of proteins released into the media. The more virulent the subculture the more evident were extracellular proteins (Figure 1). Initially it was thought that these proteins were released into the media by cell lysis. However, when a comparison was done on the protein profiles of bacterial proteins released into the medium of agar or broths, and the protein profiles of the cells disrupted by sonication, these were different (Figure 2). Had these profiles been similar, one might have concluded that the proteins in the medium were due to cell lysis. Instead, the different protein profiles suggest that proteins may have been released into the medium by the living bacterial cell.

The alteration in protein patterns and the release of proteins into the media may reflect growth stresses imposed on the bacterium. During the initial subcultures, the bacterium, initially grown in complex medium, would have had to adapt to the more restrictive synthetic salts medium. The stress of being grown in a sub-optimal medium may have caused the expression or deregulation of components associated with virulence, similar to that observed for hemolysin expression by *Vibrio parahaemolyticus* grown on synthetic media [13]. During later subcultures, the bacterium may have adapted to the medium, being able to suppress the expression of these components and hence becoming less virulent.

Characterization of an *F. tularensis* LVS 52 kDa Protein

The above results showed that proteins were present in the supernatants of the different preparations. The results also showed that these and other components were likely secreted, rather than released by lysis. To date, studies on tularemia have dealt with proteins associated with the bacterial cell grown mostly under more optimal conditions [14, 15, 16]. By using *F. tularensis* LVS grown in a suboptimal synthetic salts medium, extracellular proteins were detected that might otherwise have been missed had different growth conditions been used.

To resolve the significance of the proteins released into the media, the extracts were freeze-dried and then investigated with Western immunoblots. Initially a commercial rabbit anti-tularemia antiserum was used as the source of antibodies. The Western blots did not reveal any striking bands (data not shown). Next, filter-sterilized antiserum from mice infected with *B. abortus* was used. As *B. abortus* and *F. tularensis* cross-react [17] it was thought that mouse anti-*Brucella* serum might serve as an antibody source to identify *F. tularensis* antigens. Results were again negative (data not shown).

The cross-reaction between *Brucella* and *Francisella tularensis* was then taken a step further. A *B. abortus* O-polysaccharide vaccine that protects mice from brucellosis [18] was used to cross-protect mice from tularemia (results unpublished). Whereas unvaccinated control mice did not survive 10 LD₅₀ of *F. tularensis* LVS, 12 of 20 mice (60%) of the vaccinated mice survived this infection. The antisera from these latter mice was pooled and the antiserum used as a source of antibodies to identify *F. tularensis* LVS antigens. Upon using this antiserum on immunoblots of culture supernatants with the noted secreted proteins, antibodies did label components not evident previously. The predominant protein detected was estimated to have a molecular weight of about 52 kDa (Figure 3).

When supernatant preparations that contained the 52 kDa protein were digested with proteinase K, the band was no longer evident, showing that it was indeed a protein (Figure 4). Periodate treatment followed by silver staining also showed that the 52 kDa protein was occasionally associated with carbohydrate (data not shown). However, this was not a consistent observation. Aside from the 52 kDa protein, there were other proteins of note (Figure 4). When the concentrated preparation was digested with proteinase K, these proteins did not appear in polyacrylamide gels (Lane 3). Digestion with lipase caused the loss of a 45 kDa band (lane 5). Digestion with DNase caused the shift of a 19 kDa band to a higher 33 kDa protein, suggesting dimerization (Lane 7). Further characterization, or the possible use of these proteins as vaccine candidates against tularemia in the mouse model, has yet to be done.

It was investigated whether the noted 52 kDa protein, initially found outside the cell grown in agar or broth synthetic salts medium, could also be found inside the cell when the above mentioned antiserum was used. Usually the third broth subculture yielded the greatest amount of 52 kDa protein in the supernatant. *Francisella tularensis* LVS was subcultured (once on Chamberlain's agar medium and twice in synthetic salts broth medium), the cells were isolated with centrifugation and these were disrupted by sonication. The preparation was then standardized by diluting the protein to 3 mg/ml. Figure 5 shows that the 52 kDa protein was also found within the cell, although it appeared to increase until the culture reached stationary phase at day 5. Afterwards the protein appeared to decrease in amounts as the culture matured.

Curiously, it was also observed that the antiserum bound to the protein myosin that was in the molecular weight protein standards (data not shown). It was not determined whether this binding was due to cross-reaction between myosin and *F. tularensis* proteins, or whether, as a consequence of active tularemia, the mice had made auto-antibodies to their own myosin. In either case, anti-myosin antibodies might be a novel indirect test to identify tularemia in an animal or human.

Identification of the 52 kDa protein as a toxic virulence factor

Other researchers have studied the proteins of *F. tularensis* LVS, but to date toxins or virulence factors have not been found [7, 8]. In our study, a striking result of immunostaining with antiserum, taken from vaccinated mice that survived multiple lethal doses of *F. tularensis* LVS, was the identification of a prominent *F. tularensis* 52 kDa protein.

As a source of the 52 kDa protein for purification, *F. tularensis* LVS was grown in the synthetic salts medium, the cells were isolated by centrifugation and then ruptured by sonication. Whole cells and debris were removed from this cell lysate by centrifugation and filter-sterilization through a 0.45 µm filter. Aliquots of this preparation were saved for further studies and the rest was concentrated by filtration using an Amicon with a 30,000 m.w. cutoff filter. This concentrate was then fractioned on a Pharmacia Mono-Q column using FPLC (Figure 6). Although Fraction 24 had the greatest amount of the 52 kDa protein, as shown by SDS-polyacrylamide gel electrophoresis (Figure 7), Fraction 29 was used in further studies due to its higher purity.

When HEA cell cultures were first tested for sensitivity to this 52 kDa protein, no effect was observed. However, when Vero (Green Monkey Kidney Cells) cell cultures were used, cell death was observed, especially if the preparation of the 52 kDa protein was concentrated. The 52 kDa protein also affected cell cultures of J774A.1 cells (derived from BALB/c mouse macrophages). The test sample was standardized by the amount of protein given, and when greater than 0.5 mg/ml, cell death for both mammalian cell cultures occurred (about 50% death at 1 mg/ml, Figure 8) after 24 hr incubation. These results support the preliminary observations that *F. tularensis* produced a component that was toxic but that its effect was delayed for susceptible tissues. For protein amounts less than 0.1 mg/ml, cell death was not observed.

When mammalian cells are stressed, these may produce nitrous oxide [19]. For J774A.1 mouse macrophage cells, nitric oxide production correlated with the amount of the 52 kDa protein in the different Mono-Q fractions noted above (Figure 9). Nitric oxide also increased when protein amounts were in the sublethal range of 0.1 mg/ml to 0.5 mg/ml (Figure 10). For higher amounts of the 52 kDa protein, the cells had been killed and hence did not express nitric oxide. As the 52 kDa protein stressed the mammalian cell cultures tested to produce nitric oxide, or killed these cells at higher concentrations, our results strongly suggest that the 52 kDa protein was indeed a toxin. The previously described observation, that mice died when given 0.050 ml of *F. tularensis* LVS culture supernatant intranasally, can now be described as likely due to host cell death caused by bacterial components in the supernatant.

Vaccine/immunization studies

As time restraints allowed only a brief inspection of vaccine potential, a rapid screen was done. Two hundred and fifty, rather than 10, LD₅₀ of *F. tularensis* LVS were given as the challenge dose. It was thought that by using a much higher infectious dose that components of only marginal vaccine efficacy would be eliminated. The infection was given intranasally, rather than intraperitoneal, to reflect a biological warfare scenario of challenge. Outbred CD1 mice, rather than inbred BALB/c mice, were used to reflect genetic diversity more relevant to human populations. Mice were monitored for 3 weeks rather than 1 week to account for any delayed responses and to ensure that recovery was complete. Table 1 shows that some of these vaccines, or vaccine combinations, gave obvious protection even under these exceptionally harsh challenges.

Table 1. Assessment of different vaccine candidates for the protection of CD1 mice against 250 LD₅₀s of *F. tularensis* LVS given intranasally.

GROUP	VACCINATION (given i.p.)	PROTECTION FROM CHALLENGE (250 LD ₅₀ <i>F. tularensis</i> LVS)
1	<u>Control</u> (no vaccination, 0.1 ml sterile PBS/mouse)	0/5 mice (0%)
2	<u>Different Brucella vaccine formulations:</u> <i>B. abortus</i> 1119-3 OPS (1 µg/mouse)	2/5 mice (40%)
3	<i>B. melitensis</i> 16M OPS (1 µg/mouse)	0/5 mice (0%)
4	<i>B. suis</i> 145 OPS (1 µg/mouse)	0/5 mice (0%)
5	<u><i>F. tularensis</i> cell lysate</u> Cell lysate (2 µg/mouse)	5/5 mice (100%)
6	<u><i>F. tularensis</i> cell lysate, Amicon fractionated</u> > 30 kDa (2 µg/mouse)	1/5 mice (20%)
7	>30 kDa (2 µg/mouse) + <i>B. suis</i> OPS (1 µg/mouse)	5/5 mice (100%)
8	<u>Purified 52 kDa component (Fraction #29)</u> Fraction #29 (2 µg/mouse)	1/5 mice (20%)
9	Fraction #29 (2 µg/mouse) + <i>B. suis</i> OPS (1 µg/mouse)	2/5 mice (40%)

All control mice (Table 1, Group 1) died from this high dose of *F. tularensis* LVS, as expected. Upon studying the effects of cross-protection, different sources of *Brucella* OPS appeared to have different effects. The species of *Brucella* express different forms of the polysaccharide on the cell wall [9]. When different *Brucella* O-polysaccharide (OPS) vaccines were tested, *B. abortus* OPS (Group 2) appeared to give the best protection against tularemia in mice. However, even though *B. suis* OPS did not appear promising when used alone, when used with a high molecular weight fraction of sonicated *F. tularensis* cells, this gave total protection to the mice (Group 7).

In other publications, vaccination with whole killed *F. tularensis* cells did not give mice immunity from infection [20]. In support of this observation, when commercial rabbit antiserum (raised against whole killed cells of *F. tularensis* strain SCHU) was used, the antibodies identified little in the immunoblots of the bacterium. In our study, however, for *F. tularensis* LVS (made exceptionally virulent by growth in a synthetic salts medium, killed with phenol then lysed by sonication), this gave total protection for infected mice (Group 5).

The entire bacterial lysate may be unnecessary to protect mice from tularemia. When the lysate was fractioned to yield a preparation with components greater than 30,000 molecular weight, it gave some protection to mice from tularemia (Group 6) that was greatly enhanced when *B. suis* OPS was added (Group 7).

As our studies have shown that the 52 kDa protein was identified as a significant factor in the disease process of tularemia, mice were vaccinated with this component (Group 8). The fraction did protect mice to a minor extent and this protection was enhanced with *B. suis* OPS vaccine (Group 9). Use of other OPS, such as that from other *Brucella* species or from *F. tularensis*, might enhance the efficacy of the 52 kDa protein as a vaccine to protect mice from tularemia.

Other investigators have tested the OPS, of *F. tularensis*, linked to bovine serum albumin in the protection of mice from interdermal or aerosol challenges of tularemia [21]. Although protection was limited to a low LD₅₀ interdermal challenge, it did extend the life of mice given respiratory infections. In our study, the OPS of *Brucella* enhanced the vaccine efficacy of *F. tularensis* components in protecting mice from a very high intranasal challenge. The mechanism of *Brucella* OPS vaccine protection against respiratory *F. tularensis* was not resolved. It may have enhanced the host's immune response against the bacterium, against the 52 kDa protein (i.e. the form associated with carbohydrate) or acted as an immunomodulator that enhanced the host's general resistance to infection. Regardless of how it acted, the *Brucella* OPS vaccine appeared to enhance the effect of *F. tularensis* LVS components on protecting mice from tularemia.

As some toxins of other bacteria are encoded onto plasmids [22], an attempt was made to isolate extra-chromosomal components from *F. tularensis* LVS, grown in a synthetic broth medium and when most virulent. This was without success. Also, when a similar culture was grown but treated with chloroform (0.5 ml of chloroform for a 500 ml culture) prior to the culture reaching the stationary phase, microscopic tubular structures were present in the culture supernatant as evident by electron microscopy (data not shown). It has yet to be determined if these are a bacteriophage or simply artifacts that arose from the chloroform treatment. Although we were unable to demonstrate plasmids or bacteriophages as influencing the virulence of *F. tularensis* LVS, perhaps other investigators, using different methods, will be more successful.

Conclusions

In recent years there has been much progress in understanding the virulence mechanisms of *F. tularensis* [8, 14, 16] and working towards a protective subcellular, rather than live bacterial, vaccine [5, 15, 21]. Possibly our findings, that have resulted from a different approach, will add to these investigations.

In our study, most of the mice given a cross-protective *Brucella* OPS vaccine survived 10 LD₅₀ of *F. tularensis* LVS, made exceptionally virulent by subculture in Chamberlain's synthetic salts medium. As yet, we have not resolved why the effective vaccine against one bacterium is also cross-protective to another. We have found that both the polysaccharide and the proteins are cross-reactive with polyclonal rabbit antiserum but not with monoclonal mouse antibodies (M.L. van Hoek, J.W. Cherwonogrodzky and B.B. Brooks, data not shown). We have also found that the polysaccharide does have immuno-modulating properties (B. Kournikakis et al., US Patent 6,444,210). Whether the *Brucella* OPS vaccine offers specific immunity to cross-reacting bacteria, generic immuno-stimulation to infectious agents, or both, is secondary to the observation that it does offer protection to mice from both brucellosis and tularemia.

Within the sera of *Brucella* OPS vaccinated and *F. tularensis* challenged mice, antibodies were present that detected previously overlooked proteins. A 52 kDa protein, for example, was only expressed in trace amounts by the bacterium as evident by Coomassie Blue or silver staining of SDS-PAGE preparations but was the predominant antigen identified in immunoblots by these antibodies. Other proteins were also made more evident in immunoblots with this antiserum, but we have not yet investigated their significance. This novel approach, therefore, may provide a useful tool for investigating previously overlooked proteins or other subcellular components.

To date, there has been no evidence that *F. tularensis* produces a toxin [7, 8]. Our initial findings supported this evidence for we did not find a toxin with immediate effects on a wide range of mammalian cells. However, our later findings suggest that at least the 52 kDa protein has a delayed effect (i.e. toxicity after 24 hours) on some cell lines (e.g. Vero and J774A.1, but not HeLa). In sensitive cell lines, the production of nitric oxide is evident for low concentrations of the 52 kDa protein, and cell death occurs for high concentrations. In previous studies, we have observed that when mice were given *F. tularensis* LVS with enhanced virulence through subculture on Chamberlain's medium, lungs were the most affected tissues with foci of pneumoniae and necrosis (L.N. Schofield and J.W. Cherwonogrodzky, data not shown). It may be useful to determine if cells of the lung are sensitive to this protein, and if the symptoms of pleuropneumoniae for tularemia are a consequence of this sensitivity.

Due to the concerns and conservation of animal use, the study presented used only a few groups of mice. A pilot study was done to determine if there was any potential for subcellular components, rather than living bacteria, as vaccines. An exceptionally harsh condition was devised to screen these components (mice vaccinated with these components were given 250 LD₅₀, and this was given intranasally to cause a respiratory infection). Even under these exceptionally challenging circumstances, some fractions or combinations of antigens gave full protection. We have yet to determine if this protection is specific for *F. tularensis* LVS made exceptionally virulent, or generic, offering protection against a broad range of field strains. Hopefully these encouraging results will justify an additional, more thorough, investigation of *F. tularensis* subcellular components as vaccine candidates.

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Abbreviations

BIS - N,N'methylene-bis-acrylamide

BSL-2 - BioSafety Level 2

BSL-3 - BioSafety Level 3

cfu - colony forming units (number of bacteria)

DTT - dithiothreitol

HEPA - high efficiency particulate air (filter)

HRP - horseradish peroxidase

i.n. - intranasal

i.p. - intraperitoneal

kDa - kilodalton (molecular weight)

LD50 - 50% lethal dose (the dose that is lethal to 50% of the mice tested)

LPS -lipopolysaccharide

LVS - live vaccine strain

m.w. - molecular weight

OD - optical density

OPS - O-polysaccharide

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline (usually 0.92% in water)

TEMED- N,N,N',N'-tetramethylenediamine

TRIS -tris-hydroxymethyl-aminomethane

Patent Information

The above findings and claims have recently been filed as a Provisional US Patent Application. Any licensing or commercialization of this patent, or those related to this topic and held by DRDC, should be directed to the Business Development Officer, DRDC Suffield.

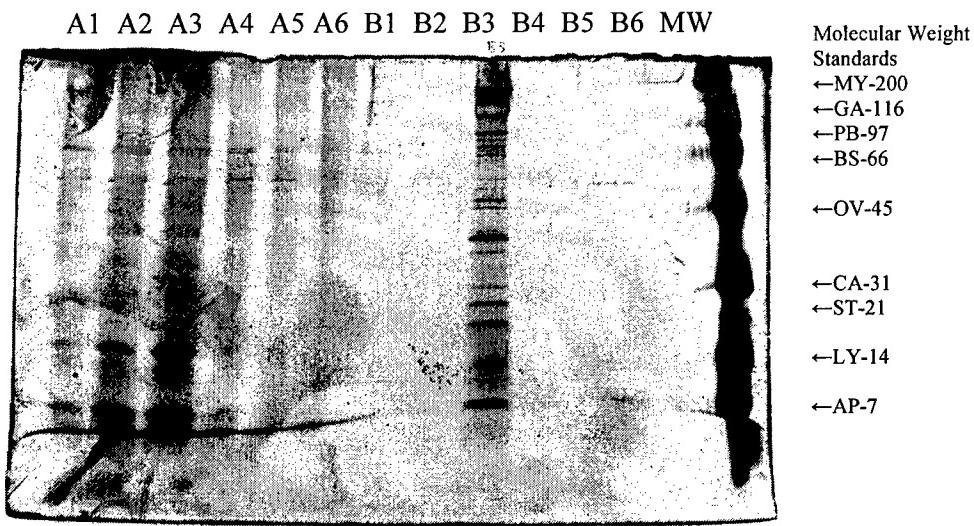


Figure 1. Silver stain of extracellular components of *Francisella tularensis* live vaccine strain sequentially subcultured on agar (A) and in broth (B) synthetic media. Cultures were sequentially subcultured daily but incubated for 2 days before inspection. For a culture grown on agar medium, the cells were scraped from the agar surface, resuspended in phosphate-buffered saline, the cells removed by centrifugation, and the liquid was freeze-dried, reconstituted then dialyzed against distilled water (extracellular components of agar subcultures are Lanes A1-A6). For cultures grown in broth medium, the cells were removed by centrifugation and the components in the supernatant concentrated as before (extracellular components of broth subcultures are Lanes B1-B6). Lane MW contains protein standards of different molecular weight: myosin heavy chain 200kDa m.w. (MY-200), β -galactosidase 116 kDa m.w. (GA-116), phosphorylase b 97 kDa m.w. (PB-97), bovine serum albumin 66 kDa m.w. (BS-66), ovalbumin 45 kDa m.w. (OV-45), carbonic anhydrase 31 kDa m.w. (CA-31), soybean trypsin inhibitor 21 kDa m.w. (ST-21), lysozyme 14 kDa m.w. (LY-14), aprotinin 7 kDa m.w. (AP-7).

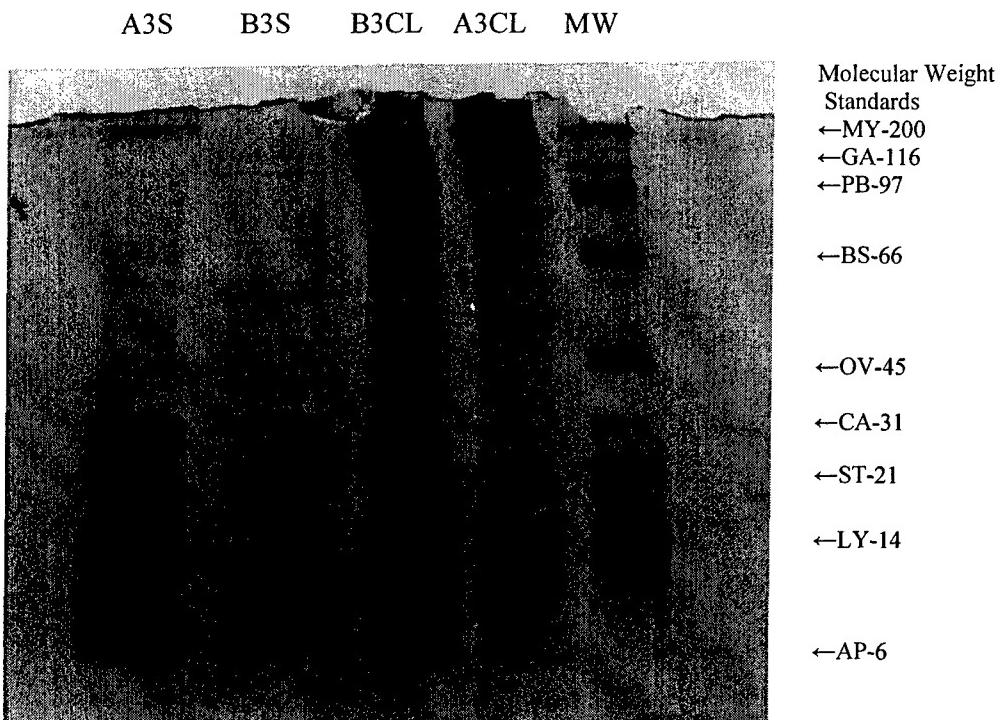


Figure 2. Silver stain of *F. tularensis* LVS culture supernatants or cell lysates. Cultures were subcultured either on agar (A) or in broth (B) synthetic salts media three times (3) and incubated for 2 days. For the former, the cells were removed from the agar, and proteins secreted into the agar were extracted into PBS then concentrated. For the latter, cells were removed by centrifugation and the proteins in the supernatant concentrated. These extracellular preparations are denoted as "S". Cells, from the noted agar or broth cultures, were disrupted by sonication and the cell lysates are denoted as "CL". The far right lane contains proteins of different molecular weight (MW, see Figure 1).

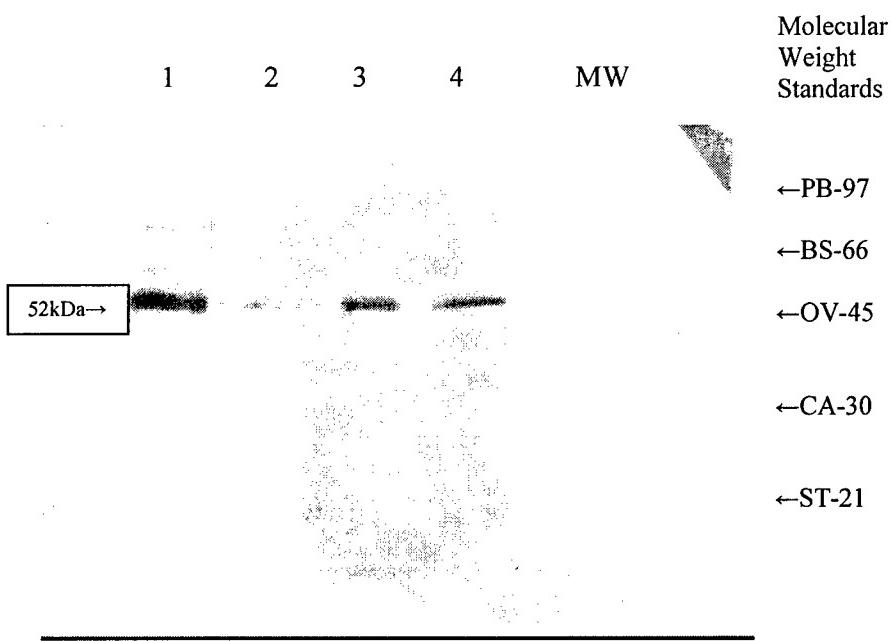


Figure 3. Immuno-staining of *F. tularensis* LVS supernatant components. The bacterium was subcultured sequentially on broth synthetic medium (cultures 1-4), incubated for 2 days and the proteins in the supernatant were concentrated as noted in the text. The proteins were separated by PAGE then transferred to nitrocellulose. A 52 kDa protein was then identified by antibodies in antiserum (from mice vaccinated with *B. abortus* O-polysaccharide and then survived 10 LD₅₀ of *F. tularensis* LVS). The far right lane contains markers of known molecular weight (MW, see Figure 1).

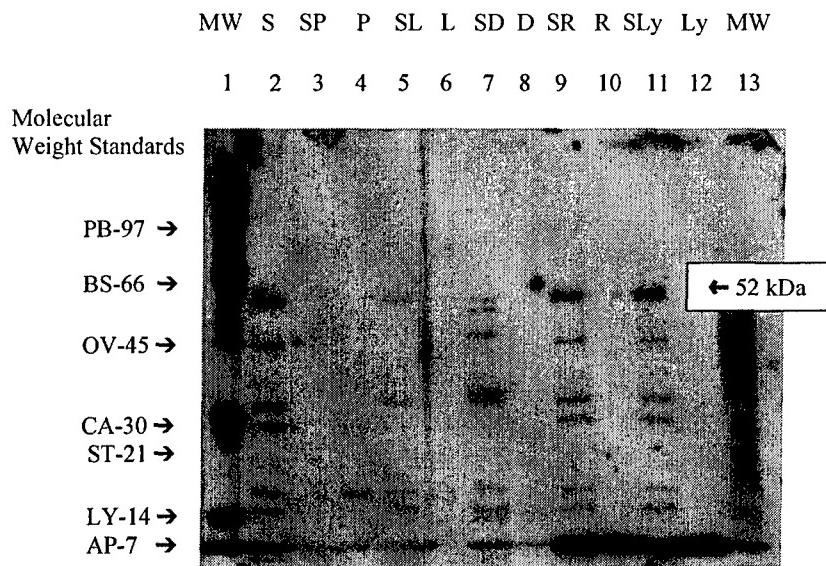


Figure 4. Enzyme digestion and immuno-staining of *F. tularensis* LVS components released into the medium of a 7 day culture grown in a synthetic salts broth. Cells were removed by centrifugation and filtration, the supernatant was collected and its components were concentrated by freeze-drying then dissolved in PBS. Supernatant samples (37.6 µg protein/0.1 ml PBS) were incubated with different enzymes (16.6 µg protein/0.1 ml PBS). After incubation (1 hr, 37°C), 10 µl was transferred to the noted respective well for SDS-PAGE, then transblotted onto nitrocellulose. The primary antibody was 1:1000 diluted Brucella OPS vaccine primed/*F. tularensis* LVS challenged mouse antiserum. The secondary antibody was 1:3000 diluted anti-mouse-HRP. Samples were: molecular weight marker (MW, Lanes 1 and 13), 7 day culture supernatant components (S, Lane 2), proteinase-K digested supernatant components (SP, Lane 3), proteinase-K (P, Lane 4), lipase digested supernatant components (SL, Lane 5), lipase (L, Lane 6), DNase digested supernatant components (SD, Lane 7), DNAse (D, Lane 8), RNAse digested supernatant components (SR, Lane 9), RNAse (R, Lane 10), lysozyme digested supernatant components (SLy, Lane 11), lysozyme (Ly, Lane 12).

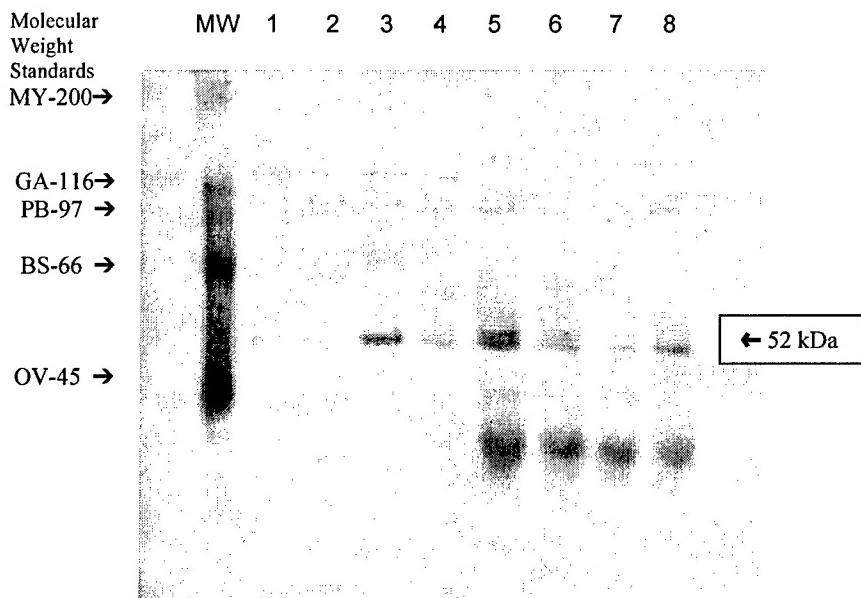


Figure 5. Immuno-staining of *F. tularensis* LVS cell lysates (sonicated). Samples were prepared and electrophoresed under native conditions then transblotted to nitrocellulose. Protein concentrations were standardized to 300 ng/well. The primary antibody source was 1:50 diluted Brucella OPS vaccine primed/*F. tularensis* LVS challenged mouse antiserum. Secondary antibody was 1:3000 diluted anti-mouse-HRP. Samples were molecular weight markers (MW) and 1-8 day broth culture cell lysates (Lanes noted as 1-8).

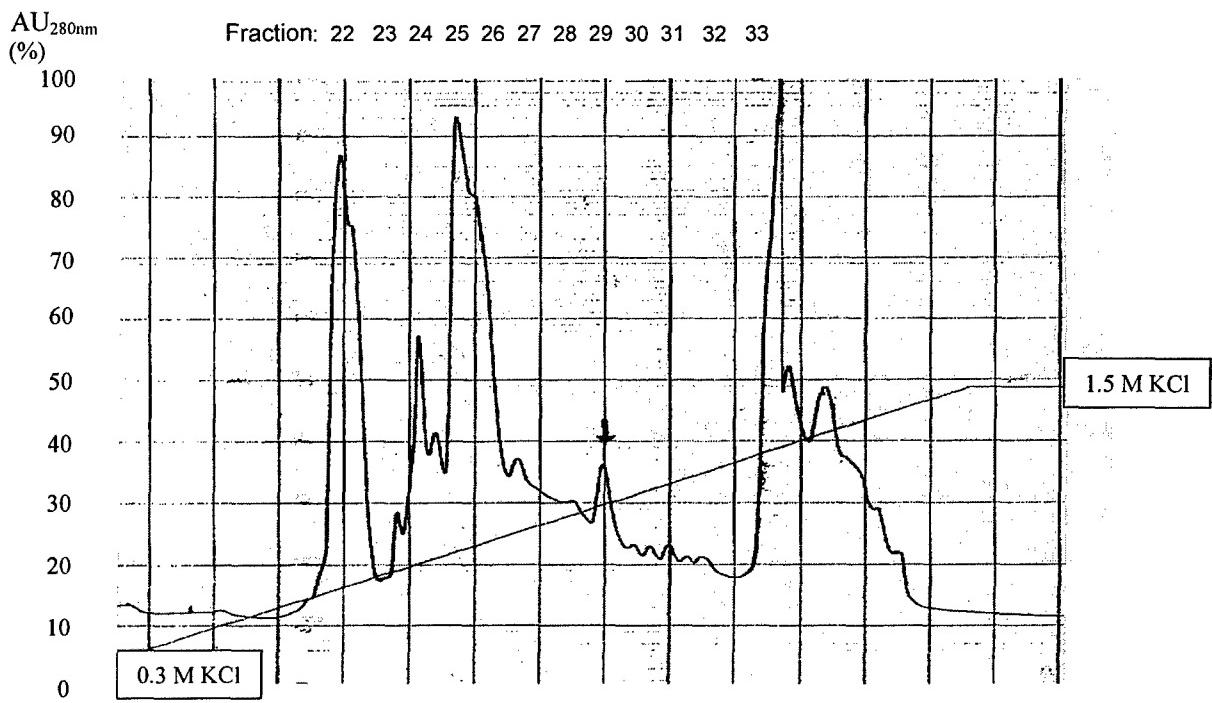


Figure 6. Elution profile (absorbance at 280 nm) of proteins secreted by *F. tularensis* LVS (second subculture in synthetic broth medium). Cells isolated by centrifugation, sonicated, partially purified by Amicon filtration, then applied to a Pharmacia Mono-Q 10/10 column. Proteins were eluted with a gradient of 0.3 to 1.5 M KCl. Eluted fractions are as noted.

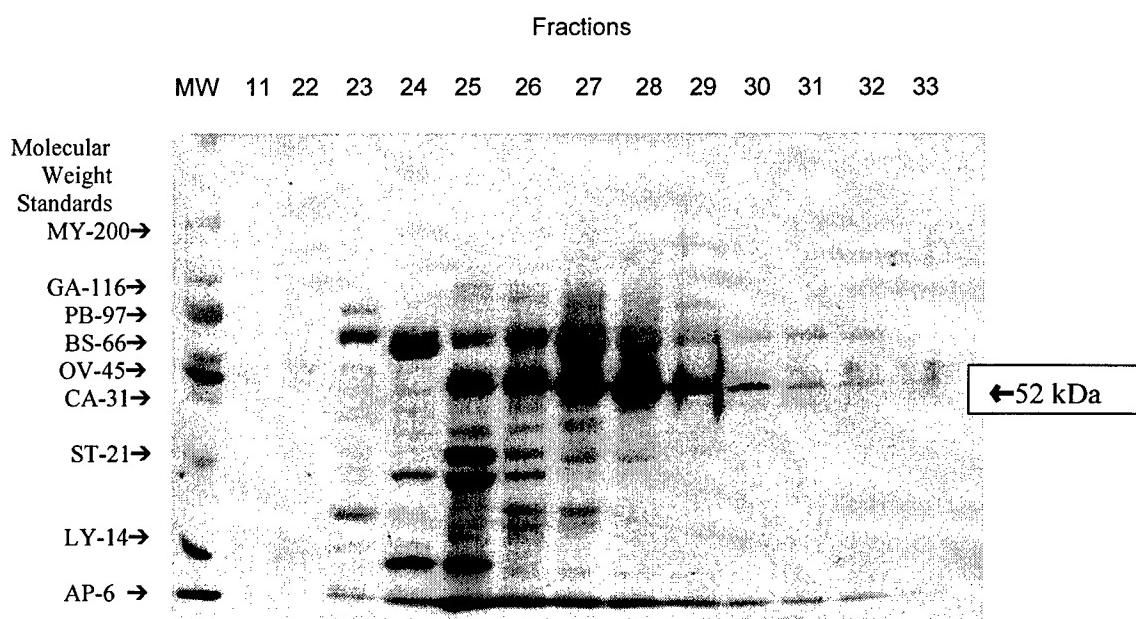


Figure 7. Immunostaining of the Pharmacia Mono-Q 10/10 fractionated *F. tularensis* LVS cellular components as noted in Figure 6. Fractions 11-33 are as noted on the figure. The molecular weight standards are as noted in Figure 1.

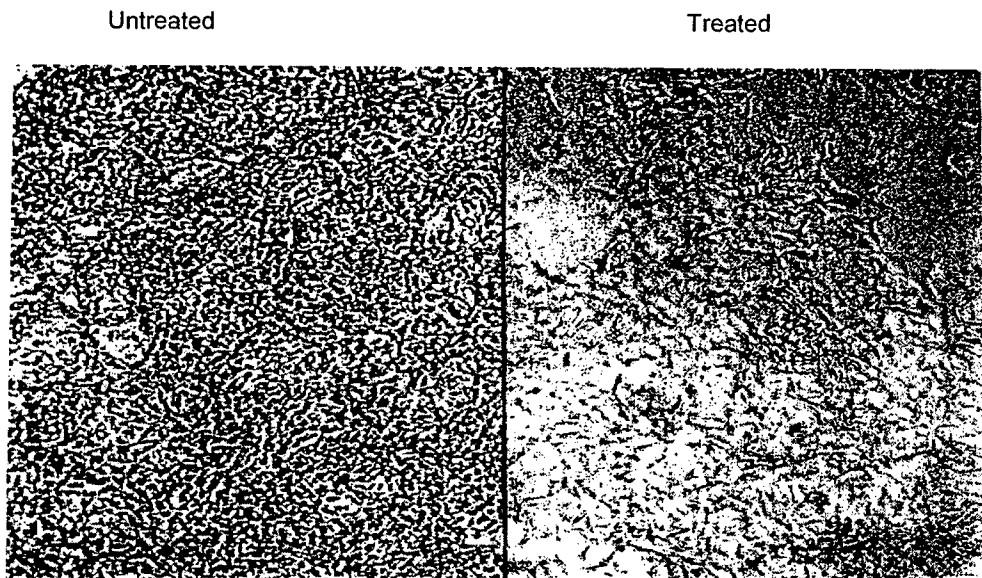


Figure 8. Vero (Green Monkey Kidney) cell culture left untreated or given 1 mg of the *F. tularensis* LVS 52 kDa protein (Fraction 29, see Figures 7 and 8) per ml of culture medium.

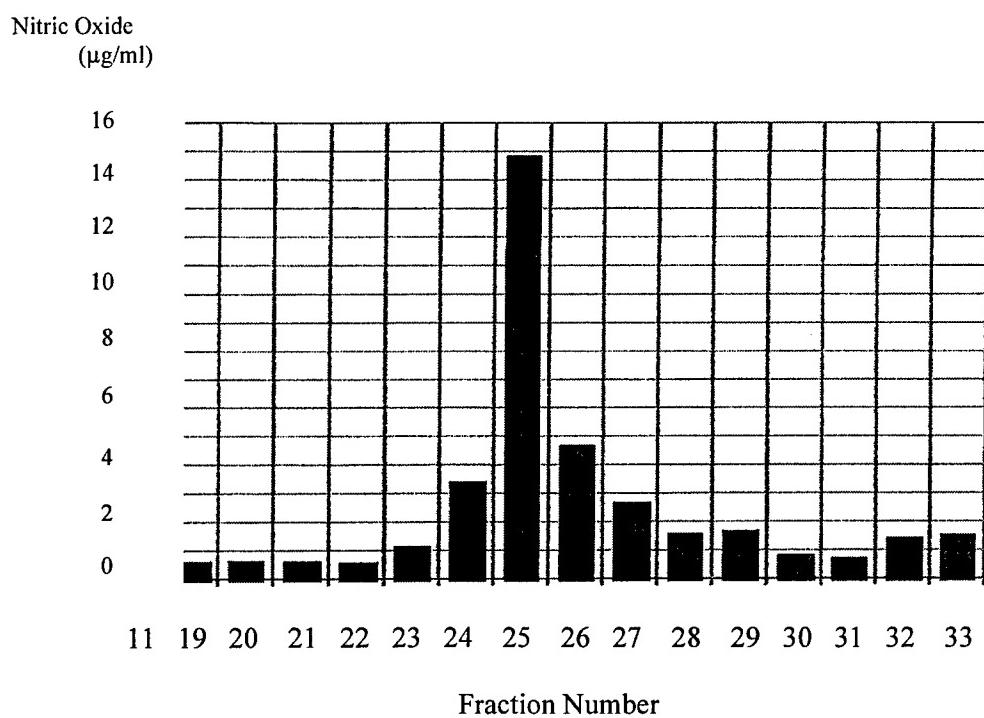


Figure 9. Nitric oxide production by J774.1 macrophage cells in response to fractions of *F. tularensis* LVS cell lysate eluted from a Pharmacia Mono-Q column.

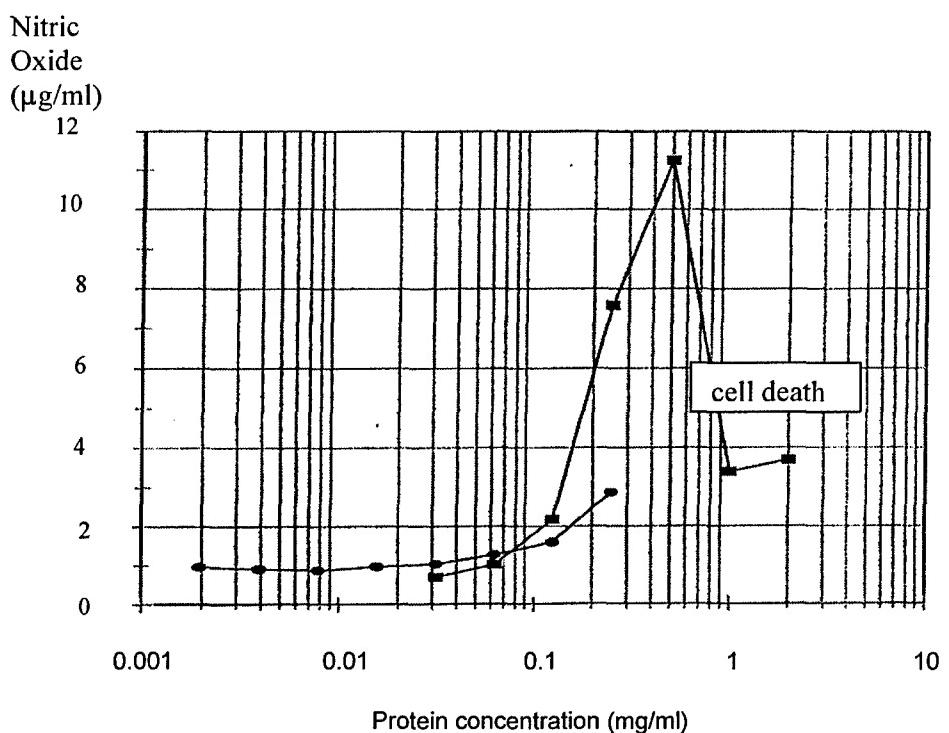


Figure 10. Nitric oxide production by J774.1 macrophage cell culture in response to treatment with the 52 kDa protein for 24 hours incubation. The protein was purified by FPLC using a Pharmacia Mono-Q 10/10 column. The source of this protein was a supernatant concentrate from a *F. tularensis* LVS primary culture (●) or secondary subculture (■).

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For identifying *Francisella tularensis* vaccine candidates, mice were first vaccinated with *Brucella abortus* O-polysaccharide (OPS) vaccine. These animals were then given 10 LD₅₀s of *F. tularensis* live vaccine strain (LVS). Sixty percent (60%) of the vaccinated mice survived the multiple lethal dose while all the unvaccinated control mice perished. Sera were collected from these surviving mice and used to probe supernatant and cell lysates of live *F. tularensis* LVS cultures. Several *Francisella tularensis* components were identified by this noted antiserum. Mouse serum from mice vaccinated with killed *F. tularensis* did not identify these components. Of these identified proteins, enzyme digestions and chemical oxidation suggest post-translational modifications for some of the proteins (e.g. a 52 kilodalton (kDa) glycoprotein, a 45 kDa lipoprotein and a 19 kDa nucleoprotein). In low concentrations, the 52 kDa component caused nitrous oxide induction in tissue cultures and in high concentrations it caused cell death. Vaccination with this protein gave mice partial protection (20% survival) from 250 LD₅₀ of tularemia given intranasally while the addition of other components may have acted synergistically to give enhanced protection (i.e. 100% survival).

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brucellosis
vaccine
polysaccharide
protein
LD₅₀
Surrogate marker
intranasal
mice

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